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(54) Title: EXPRESSION OF PROTEINS FROM AMPLIFIED, IMMOBILIZED NUCLEIC ACIDS

(57) Abstract: A protein is made by immobilizing a PCR primer onto a solid support, then using the immobilized PCR primer along with a soluble primer to amplify and immobilize a template DNA containing a protein coding sequence onto the solid support. The template DNA and/or the PCR primers also contain regulatory sequences for transcription and translation of the coding sequence. The immobilized DNA is then transcribed and translated to produce the protein. The immobilized DNA may be reused for multiple cycles of transcription and translation. By immobilizing a universal PCR primer onto the solid support, different template DNAs can be amplified and immobilized and a number of different proteins can be made at the same time. If particles are used as the solid support, the particles may be injected into an organism so that the steps of transcription and translation take place within the organism to produce a protein vaccine or therapeutic.

1 **EXPRESSION OF PROTEINS FROM AMPLIFIED, IMMOBILIZED NUCLEIC ACIDS**

2

3 **Technical Field**

4 The present invention relates to the production of proteins generally and in particular to the

5 production of proteins using DNA that is immobilized and amplified onto a solid surface by means

6 of an immobilized oligonucleotide primer.

7

8 **Background Art**

9 The polymerase chain reaction (PCR) is a powerful tool that enables logarithmic

10 amplification of DNA sequences, resulting in a 10^6 - 10^8 fold enhancement of a target sequence. The

11 amplified DNA can be used for a variety of downstream experimental or diagnostic purposes, such

12 as the detection of a minute amount of a particular DNA sequence in a sample. The basics of PCR

13 are described, for example, in the following U.S. patents: U.S. Patent No. 4,683,195 to Mullis et al

14 for "Process for Amplifying, Detecting and/or Cloning Nucleic Acid Sequences", U.S. Patent No.

15 4,683,202 to Mullis for "Process for Amplifying Nucleic Acid Sequences" and U.S. Patent No.

16 4,800,159 to Mullis et al for "Process for Amplifying, Detecting and/or Cloning Nucleic Acid

17 Sequences".

18 As efficient as the PCR process can be, recovery of amplification products for subsequent

19 uses can involve tedious purification procedures such as gel electrophoresis, organic extraction,

20 centrifugation, and/or column purification. Consequently, methods have been developed for creating

21 immobilized PCR amplicons by immobilizing a PCR primer onto a solid substrate. Such methods

22 are described, for example in the following patents and publications: Kohsaka et al "Solid-Phase

23 Polymerase Chain Reaction", Journal of Clinical Laboratory Analysis 8 (1994), pp 452-455;

24 Rasmussen et al, "Combined Polymerase Chain Reaction-Hybridization Microplate Assay Used to

25 Detect Bovine Leukemia Virus and *Salmonella*" Clinical Chemistry, vol. 40, No. 2 (1994), pp 200-

26 205; Oroskar et al, "Detection of Immobilized Amplicons by ELISA-like Techniques" Clinical

27 Chemistry vol. 42, No. 9 (1996) pp 1547-1555; Lockley et al "Colorimetric Detection of

28 Immobilised PCR Products Generated on a Solid Support" Nucleic Acids Research vol 25, No. 6

29 (1997) pp 1313-1314; U.S Patent No. 5,922,574 to Minter for "Method for Producing Copies of a

30 Nucleic Acid Using Immobilized Oligonucleotides"; and U.S. Patent No. 5,910,406 to Minter for

31 "Manipulating Nucleic Acid Sequences". Typically, immobilized PCR primers are used in

32 diagnostic assays to detect the presence of minute amounts of specific DNAs in a sample.

1   In a separate field of technology, methods have been developed for *in vitro* synthesis of

2   proteins by cell-free transcription and translation of protein-encoding DNA. Cell-free synthesis of

3   proteins is disclosed, for example, in the following U.S. patents: U.S. Patent No. 5,556,769 to Wu

4   et al for "Coupled Replication-Translation Methods and Kits for Protein Synthesis"; U.S. Patent No.

5   5,492,817 to Thompson et al for "Coupled Transcription and Translation in Eukaryotic Cell-Free

6   Extract"; U.S. Patent No. 5,665,563 to Beckler for "Coupled Transcription and Translation in

7   Eukaryotic Cell-Free Extract"; and U.S. Patent No. 5,895,753 to Mierendorf et al for "Method for

8   In Vitro Protein Synthesis". Coupled methods of DNA amplification and protein synthesis are

9   described, for example, in the following U.S. Patents: U.S. Patent No. 5,571,690 to Hecht for

10   "Method for the Cell-Free Synthesis of Proteins" and U.S. Patent No. 5,807,717 to Joyce for

11   "Coupled Isothermal Polynucleotide Amplification and Translation System". Typically, the

12   amplified DNA used in the production of the protein is not recoverable or reusable, but rather is

13   discarded during the process of purifying the protein.

14   A method for producing RNA from immobilized DNA templates is described in U.S. Patent

15   No. 5,700,667 to Marble et al for "Strategy for the Production of RNA from Immobilized

16   Templates".

17   In another area of technology, DNA vectors have been developed for genetic vaccines and

18   genetic therapeutics. In a technique known as genetic immunization, a DNA vector containing a

19   coding sequence for a protein antigen as well as appropriate regulatory sequences is injected into an

20   organism. Immunization can be by way of intramuscular, intravenous or intra-nasal routes. Inside

21   the organism, the DNA is transcribed into RNA and the RNA is translated into the protein antigen.

22   The protein antigen then triggers an immune response in the organism, typically activating both the

23   humoral and cellular immune responses. Methods of genetic immunization are described, for

24   example, in the following U.S. patents: U.S. Patent No. 5,589,466 to Felgner et al for "Induction of

25   a Protective Immune Response in a Mammal by Injecting a DNA Sequence"; U.S. Patent No.

26   5,739,118 to Carrano et al for "Compositions and Methods for Delivery of Genetic Material"; U.S.

27   Patent No.5,795,872 to Ricigliano et al for "DNA Construct for Immunization"; U.S. Patent No.

28   5,814,617 to Hoffman et al for "Protective 17 KDA Malaria Hepatic and Erythrocytic Stage

29   Immunogen and Gene"; U.S. Patent No. 5,830,876 to Weiner et al for "Genetic Immunization"; and

30   U.S. Patent No. 5,817,637 to Weiner et al for "Genetic Immunization". In genetic therapy, a DNA

31   construct with appropriate regulatory and protein coding sequences is injected into an organism to

32   produce a therapeutic protein. Typically, the purification steps required with present methods of

1   production of DNA for gene vaccines and gene therapy are extensive and time-consuming. These

2   approaches may be subject to great scrutiny by the FDA due to the presence of xenobiotic (e.g.

3   bacterial or viral) DNA, and the possible presence of endotoxins resulting from plasmid synthesis

4   in bacteria.

5

6   **Disclosure of Invention**

7   It has now been discovered the steps of transcription and translation in protein synthesis can

8   be carried out using DNA (containing the necessary coding and regulatory sequences) that is

9   immobilized on a solid support. Further, it has been discovered that the method by which the DNA

10   is immobilized onto the solid support has a dramatic effect on the efficiency of thermal cycling

11   amplification as well as subsequent transcription and translation reactions. Immobilized DNA that

12   has a density and a thermal stability optimized for amplification and subsequent transcription and

13   translation reactions can be created by first covalently attaching an oligonucleotide primer, such as

14   a PCR primer, to a solid support and then using the oligonucleotide primer in an amplification

15   reaction, such as the polymerase chain reaction, to amplify the desired DNA. Additional stability

16   may be gained by incorporating non-natural chemistries such as phosphorothioate linkages in the

17   terminal sequences of at least one of the second oligonucleotide primers.

18   Accordingly, the present invention is directed to a method of making a protein comprising

19   the steps of:

20   (a) providing

21   (i) a solid support,

22   (ii) a template DNA that includes at least one sequence that encodes a protein, and

23   (iii) first and second oligonucleotide primers, wherein the first oligonucleotide

24   primer, the second oligonucleotide primer and/or the template DNA include at least

25   one transcription regulatory sequence and at least one translation regulatory

26   sequence, and wherein the first oligonucleotide primer is covalently attached to the

27   solid support,

28   (b) amplifying the template DNA using the first and second oligonucleotide primers to

29   obtain amplicons covalently attached to the solid support, wherein the amplicons include the

30   transcription and translation regulatory sequences and the protein coding sequence,

31   (c) transcribing the covalently attached amplicons to obtain an RNA segment that includes

32   at least one protein coding sequence and at least one translation regulatory sequences, and

1   (d) translating the RNA segment to obtain the protein.

2   The present invention further includes a device for making a protein, the device comprising

3   (i) a solid support, and (ii) a DNA segment having a first end and a second end, wherein the first

4   end is covalently attached to the solid support, wherein the second end contains phosphorothioate

5   linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one

6   transcription regulatory sequence and at least one translation regulatory sequence.

7   The method of synthesis of proteins using immobilized DNA according to the present

8   invention has advantages over methods of synthesis of proteins using unbound DNA in that the

9   present method allows for DNA to be separated from a reaction mixture after the synthesis is

10   completed and to be reused in subsequent rounds of transcription and translation. Separation can

11   be easily accomplished by washing or, if the DNA is immobilized onto particles, by centrifugation

12   or by magnetic separation. Separation and reuse of DNA is much less practicable if DNA is not

13   immobilized on a solid support. Another advantage of the present invention is that the step of

14   obtaining an amplified, protein-coding sequence is simplified by the approach of using an

15   immobilized primer to simultaneously amplify and capture the amplicon, which eliminates many

16   purification steps. Still another advantage of the present invention is that multiple amplicons can be

17   amplified and immobilized from a single PCR reaction, and from these, multiple proteins may be

18   simultaneously prepared. Still another advantage is that the beads may be derivatized to target

19   immobilized DNA sequences to specific cell types or tissues.

20   The method of immobilizing DNA onto a solid support by covalently attaching a PCR primer

21   and then extending the primer through the polymerase chain reaction has been found by the

22   inventors to provide a more favorable attachment of DNA than a method of first amplifying DNA

23   and then attaching the amplified DNA to a solid support. Moreover, for carrying out the method of

24   the invention, solid supports with "universal" PCR primers (that is, primers that recognize a variety

25   of template targets) can be prepared in advance.

26   A further aspect of the invention is that DNA that is covalently attached to particles could

27   be injected into an organism as a genetic vaccine or genetic therapeutic. Transcription and

28   translation to produce a protein would then take place within the organism. It is expected that

29   coding and regulatory sequences of DNA that is covalently attached to particles will stand a better

30   chance of being transferred intact into an organism than naked DNA or DNA that is merely

31   physisorbed onto particles. It is also expected that DNA prepared according to the method described

32   by the inventors will be composed of minimal xenobiotic (to human) DNA and minimal endotoxin

1   contamination. DNA that is covalently attached to particles can be protected from enzymatic

2   degradation in an organism by incorporating chemically modified internucleotidic linkages such as

3   phosphorothioate into the second PCR primer. This is not possible with conventional plasmid

4   techniques.

5   Another advantage of the present invention is that it provides a way to synthesize several

6   different proteins at one time in a single reaction mixture. This may be done, for example, by

7   immobilizing several different PCR primers to the solid support and then amplifying several

8   different template DNAs onto the solid support and transcribing and translating each of these

9   simultaneously. It may also be done by selecting a first PCR primer that recognizes several different

10   template DNAs, then simultaneously amplifying and immobilizing the several different DNAs onto

11   the solid support, then transcribing and translating each of these simultaneously. If particles such

12   as beads are used as the solid support, different template DNAs could be amplified separately onto

13   particles and then the different particles could be combined into a single reaction mixture for

14   transcription and translation.

15

16

17   **Best Modes for Carrying Out the Invention**

18   As used herein, the term "solid support" refers to any surface or particle to which

19   oligonucleotides may be attached. Preferably, the solid support is in the form of particles, such as

20   glass particles, controlled porous glass particles, magnetic particles, polymeric particles or ceramic

21   particles. Preferably, the particles are small enough so that they can be kept suspended in an

22   aqueous solution with gentle agitation and are dense enough so that they can be easily recovered

23   from a reaction medium. Typically, the particles will have a particle size of about 50 nanometers

24   to 10 mm along an axis of greatest length. The particles may be any shape or form, including

25   spherical or irregular three-dimensional shapes or two-dimensional shapes. Commercially available

26   controlled porous glass beads or polymer beads such as are commonly used for solid phase organic

27   synthesis are suitable as solid supports for the present invention.

28   The solid support may also be a portion of the surface of a membrane, filter, test tube,

29   microtiter well, microscope slide or other surface or may be the surface of a material such as a

30   hydrogel or sol-gel.

31   As discussed below, the solid support may include any surface modification that enables the

32   first oligonucleotide primer to be covalently attached to the solid support.

5

1   As used herein, the term "protein" refers to any peptide or polypeptide that can be produced

2   by the expression of DNA. The term includes complete functional proteins such as enzymes,

3   hormones and antibodies, etc., fragments of complete proteins, fusions of two or more complete

4   proteins or fragments, and fusions of complete proteins or fragments with additional peptide

5   sequences.

6   As used herein, the term "template DNA" refers to a segment of DNA that includes a

7   nucleotide sequence that encodes a protein and may also include regulatory sequences operatively

8   linked for directing the expression, including transcription and translation, of the coding sequence.

9   The template DNA may be produced by DNA synthesis or derived from expression vectors,

10   plasmids, cosmids, genomic DNA or RNA (or a subset thereof), PCR amplicons, amplicons derived

11   from other enzymatic or non-enzymatic means, intact or lysed bacteria, viral plaques, or cells. The

12   particular coding sequence is, of course, selected according to the particular protein that is to be

13   made. The regulatory sequences are selected according to the particular expression system (bacterial,

14   mammalian, insect, yeast, etc.) to be used for expressing the DNA. Examples of transcriptional

15   regulatory sequences include promotors for T7 RNA polymerase, SP6 RNA polymerase,

16   cytomegalovirus, adenovirus, adenoassociated virus, lentiviruses, poxviruses, baculovirus and yeasts

17   such as Saccharomyces. It is not necessary, however, that entire regulatory sequences be contained

18   in the template DNA. Some regulatory sequences necessary for expression may be located initially

19   in the first PCR primer or the second PCR primer. These regulatory sequences would then become

20   operatively linked to the coding sequences when the first oligonucleotide primer and second

21   oligonucleotide primer are extended in the nucleic acid amplification reaction to form the

22   immobilized amplicons.

23   As used herein, the terms "first PCR primer" and "second PCR primer" refer to

24   oligonucleotides that are selected according to conventional PCR techniques to hybridize on

25   opposite ends and opposite strands of the template DNA. As described below, the first PCR primer

26   is covalently attached to a solid support (and is also provided in low concentration as a soluble first

27   primer) and the second PCR primer is soluble. By referring to "PCR" primers, it is not meant to limit

28   the method of the invention to amplification by PCR in its strictest and most precise definition. As

29   used herein, the terms "first PCR primer", "second PCR primer" and "polymerase chain reaction"

30   can be used interchangeably with the terms "first oligonucleotide primer", "second oligonucleotide

31   primer" and "amplification reaction" to refer to PCR or similar methods that use primer recognition

32   of a template DNA and primer extension to achieve DNA amplification or ligation of two or more

1   nucleic acid sequences.

2   Both the first PCR primer and the second PCR primer may contain additional sequences

3   besides those that are strictly necessary to effect a PCR amplification of the template DNA, and

4   these additional sequences would, during the PCR process, be incorporated into the immobilized

5   amplicons. For example, the first PCR primer or the second PCR primer may contain regulatory

6   sequences that affect the transcription and translation of the coding sequences of the template DNA.

7   The second PCR primer may contain modifications to its 5' end to make the amplicon more resistant

8   to enzymatic degradation. For example, the 5' end of the second PCR primer may include

9   phosphorothioate or methyl phosphonate-modified nucleotides.

10   In the Examples described below, the bacteriophage T7 transcriptional promoter and

11   termination sequences were used as primers since the T7 promoter allows for high levels of tightly

12   regulated transcription and thus may be useful for recombinant cloning and protein production

13   applications. See, for example, Fujita, K., and Silver, J. (1993) *Biotechniques*, 14, 608-617. With

14   design of appropriate primers, however, it is anticipated that this method would work equally well

15   for other bacterial, viral or mammalian expression systems.

16   For the steps of solid stage amplification and transcription to be carried out efficiently and

17   effectively, the first PCR primer must be coupled to the solid support with a bond that is stable

18   enough to hold up through the process of thermal cycling during PCR amplification. Covalent

19   bonding of the first PCR primer to the solid support is required. In particular, it has been discovered

20   in the present invention that many currently known methods of attaching oligonucleotides to a solid

21   support are insufficient to provide a stable enough attachment. For example, a method of covalent

22   attachment of DNA to a solid support described by Chrisey, et al, Nucleic Acids Research (1996),

23   24, 3131-3039 and US Patent 5,688,642 to Chrisey, et al, utilized a sulfhydryl-terminated

24   oligonucleotide to react with an aminated surface which had been treated with a hetero-bifunctional

25   crosslinker containing N-hydroxysuccinimide and iodoacetamide groups, to produce a thioether

26   linkage. This covalent attachment scheme was not sufficiently heat stable to be useful for an

27   amplification strategy that depends on thermal cycling. Another method that was sub-optimal

28   utilized a commercially available "oligo affinity" DNA synthesis support, which is designed to allow

29   synthesis of a DNA sequences onto a support from which it cannot be readily cleaved. This

30   approach produced sequences that lacked fidelity through normal levels of synthesis failure;

31   moreover, the density of the immobilized primers was sub-optimal.

32   For extremely long amplicons, such as amplicons with more than 5000 bases, it may be

1   desirable to prepare the amplicon separately, purify it, and then couple to a solid support in

2   preparation for transcription/translation, due to the difficulty, low yields and special conditions

3   required for fidelity in amplifying such sequences.

4   Preferably, to create a heat-stable attachment of the first PCR primer with the solid support,

5   the solid support is first modified with a silane layer that includes available reactive amine groups.

6   For example, the surface of the support may be silanized using N-(2-aminoethyl)-3-

7   aminopropylmethyl-dimethoxy-silane (EDA, Gelest, Tullytown, PA) or N-(6-aminohexyl)-

8   aminopropyltrimethoxysilane (HDA, Gelest).   The first PCR primer may also be modified to

9   include reactive amine or phosphate groups. The solid support may then be coupled to the first PCR

10   primer by carbodiimide chemistry. For example, a phosphorylated primer may be coupled to an

11   amino-functionalized surface using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-HCl (EDC,

12   Sigma Chemical Co, St. Louis, MO) in a 1-methylimidazole (Sigma) buffer to generate an amide-

13   coupled primer.

14   The covalent attachment of the first PCR primer to the solid support may also be achieved

15   by synthesizing the primer directly on the support by automated DNA synthesis using a non-

16   cleavable synthesis support.

17   Because the steps of PCR amplification and transcription require the accessibility of enzymes

18   and reactants to the individual molecules of the first PCR primer, it is preferable that the first PCR

19   primer be attached to the solid support by means of a tether or spacer such as a heterobifunctional

20   or homobifunctional crosslinker.   The crosslinker is selected based on the specific chemical

21   functionalities of the surface and the primer to be tethered. To attach an oligonucleotide to a solid

22   surface by means of a crosslinker, the most reactive end of the crosslinker is generally reacted first.

23   For example, crosslinkers bearing a succinimide ester are susceptible to hydrolysis, thus this end of

24   the crosslinker would be reacted with an amine-functionalized surface or DNA first, to maximize

25   crosslinking. The crosslinker is selected to obtain a thermally- and chemically stable linkage, such

26   as an amide bond, while avoiding those which would generate disulfides or thioethers.   The

27   synthesis of DNA is so flexible almost any reactive group can be stably incorporated. Other design

28   considerations in selection of crosslinker are length and flexibility, which may influence the

29   accessibility of the DNA to enzymes and nucleotides involved in transcription.

30   The step of amplifying the template DNA by the polymerase chain reaction using the first

31   and second PCR primers to obtain amplicons covalently attached to the solid support may be carried

32   out by any known method of PCR or similar methods, including enzymatic and non-enzymatic

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1   methods, methods that involve thermal cycling and methods that are isothermal. It is to be
2   understood that the step is carried out in the presence of any reagents and materials useful or
3   necessary for carrying out PCR including, for example, deoxynucleotide triphosphates, enzymes,
4   buffer salts, and proteins such as BSA or gelatin. The reaction medium may also include a soluble
5   version of the first PCR primer to act as a spike in promoting the PCR reaction.

6   As used herein, the term "amplicon" refers to the entire stretch of DNA that is immobilized
7   onto the solid surface, including sequences contained in the first PCR primer, the second PCR
8   primer, the template, as well as any sequences that may be attached to the solid surface before the
9   first PCR primer is attached. However, the term "transcribing the covalently attached amplicons"
10   is not meant to imply that the entire length of the amplicon is transcribed. The portion of the
11   amplicon that is transcribed is a section that contains the coding sequence and the translation
12   regulatory sequence. The selection of the portion of the amplicon to be transcribed is typically
13   governed by the transcription regulatory sequence

14   If particles are used as the solid support, then following the amplification step, the particles
15   may be recovered by separation techniques including, but not limited to centrifugation, filtration,
16   magnetic separation, or gravity separation.

17   The step of transcribing the immobilized amplicons to obtain a protein-coding RNA segment
18   and the step of translating the RNA into a protein may be carried out by any method known in the
19   art for expressing a protein from a DNA source. Preferably, the steps of transcription and translation
20   are carried out in a single reaction vessel (a "single pot" process) in an expression medium that
21   contains the necessary enzymes and reactants for producing RNA transcripts and for translating the
22   transcripts into a protein. Kits for *in vitro* transcription and translation of DNA are commercially
23   available. Commercially available kits include, for example, the Ambion mMessage Machine,
24   Boehringer Mannheim Linked *in vitro* SP6/T7 Transcription/Translation kit and mammalian
25   expression systems such as rabbit reticulocytes and canine kidney cells.

26   After the production of a protein, the protein can be separated from the reaction medium by
27   conventional methods. For example, the proteins may be synthesized as fusions with hexhistidine
28   moieties, which enable purification by chelation onto nickel columns; or as fusions with numerous
29   other proteins for which affinity purification methods exist (maltose binding protein, protein A,
30   protein G, etc). The proteins may also be synthesized using a biotinylated amino acid, which can
31   facilitate recovery using a streptavidin affinity support. Alternatively, the product protein may be
32   isolated using HPLC or similar chromatography methods.

1   After the steps of transcription and translation are carried out one time, the solid support

2   may be reused for subsequent cycles of transcription and translation. If the solid support is in the

3   form of particles, the particles may be recovered from the reaction medium using separation

4   techniques including, but not limited to centrifugation, filtration, magnetic separation, or gravity

5   separation.

6   According to a variation of the method of the present invention, several different proteins

7   may be produced in one reaction mixture. This may be done by selecting a first PCR primer that

8   recognizes several different template DNAs, each different template DNA having a coding sequence

9   for a different protein. The primer would be immobilized onto a solid support as described above

10   and then, in the amplification step, the solid support would be exposed to the several different

11   template DNAs, along with soluble primers. PCR amplification would proceed as described above,

12   and the end result would be that different amplicons (having coding sequences for different proteins)

13   would all be immobilized on the solid support. The steps of transcription and translation would be

14   carried out as described above, with the end result that different RNAs would be produced

15   simultaneously and these would be used to simultaneously produce different proteins. If the

16   amplicons are designed to include different regulatory sequences controllable by external factors,

17   the timing and relative amount of each protein that is produced could be controlled.

18   Another way to make different proteins at one time is to immobilize different first PCR

19   primers that each recognize a different template DNA and then use PCR with different template

20   DNAs and different second primers to create a solid surface having different amplicons immobilized

21   on it.

22   Another way to make different proteins at one time is to carry out the immobilization and

23   amplification steps of the invention separately on different sets of particles with different first PCR

24   primers and second PCR primers and different template DNAs and then mix the different sets of

25   particles together before carrying out transcription and translation.

26   In another variation of the present invention, a genetic vaccine or genetic therapeutic may

27   be created by immobilizing amplicons onto particles, by the method of PCR with an immobilized

28   primer as described above, combining the particles with a pharmaceutically acceptable carrier and

29   then injecting the particles into an organism so that the steps of transcription and translation take

30   place within the organism. In this variation, the template DNA would be selected to produce a

31   protein antigen or protein therapeutic, and regulatory sequences would be selected so that the

32   immobilized DNA is expressed within the particular organism. For example, pneumatic epidermal

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1   administration, a method that is currently used for injecting microparticles having physisorbed DNA,

2   may be used with particles of the present invention. An advantage of the present invention is that

3   the introduction of xenobiotic DNA into a patient is minimized, since selectable markers such as

4   markers for antibiotic resistance commonly found in bacterial plasmids are not required. Moreover,

5   pharmaceutical preparations based on the present invention can be predicted to lack pyrogens and

6   endotoxins, since the protein-encoding DNA is produced chemically and not by way of plasmid

7   replication inside bacterial cells.

8   Having described the invention, the following examples are given to illustrate specific

9   applications of the invention, including the best mode now known to perform the invention. These

10   specific examples are not intended to limit the scope of the invention described in this application.

11

12   **SPECIFIC EXAMPLES**

13   **MATERIALS AND METHODS**

14   **DNA synthesis**

15   DNA oligomers were prepared using a PE Applied Biosystems Model 394 DNA/RNA

16   synthesizer (Foster City, CA) using conventional cyanoethyl (CE) phosphoramidites; an exception

17   to this is described below under Oligo Affinity Supports.   All synthesis chemicals,

18   phosphoramidites, and chemical modifiers were obtained from Glen Research (Sterling, VA).

19   Phosphate or disulfide groups were introduced at the 5' -end of oligos using Chemical

20   Phosphorylation Reagent I or Thiol Modifier C6 S-S, respectively (Glen Research), following the

21   manufacturer's protocols. Specific details for the coupling of modified oligomers to various

22   supports are given below. Primers for the bacteriophage T7 promoter region (T7Prom: 5'-TAG-

23   GGC-GTG-AGT-CGT-ATT- AAA-ATT-AAT-ACG-ACT-CAC-TAT- AGG-GAG-A-3') and T7

24   termination site (T7Term: 5'-CAA-GGG-GTT-ATG- CTA-GTT-ATT-GCT-CAG-CGG-3') were

25   prepared and used as either soluble or bead-immobilized PCR primers. The DNA oligomers were

26   purified and detritylated using C-18 SPE cartridges (Supelco, Bellefonte, PA), and then quantified

27   as described in Chrisey, L.A., Lee, G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, **24**, 3131-

28   3039. The following **Table I** details the specific modifications made to these basic sequences.

1 **Table 1.** Summary of primer modifications
2
3

Primer	5' Chemical Modification	Crosslinker	Bead type	Text Abbreviation
T7Term	None	None	None	T7 Term
T7Term	biotin	None	avidin-MPG	B-MPG
T7Term	phosphorylation	None	EDA-modified-CPG	PC-E
T7Term	phosphorylation	None	HDA-modified-CPG	PC-H
T7Term	C6-thiol	SIAB	EDA-modified-CPG	E-SIAB
T7Term	C6-thiol	SIAB	HDA-modified-CPG	H-SIAB
T7Term	C6-thiol	NHS-PEG-MAL	EDA-modified-CPG	E-PEG
T7Term	C6-thiol	NHS-PEG-MAL	HDA-modified-CPG	H-PEG
T7Term	uncleavable support	None	OAS-PS	T-PS
T7Term	uncleavable support	None	OAS-CPG	T-CPG
T7Prom	None	None	None	T7Prom
T7Prom	uncleavable support	None	OAS-PS	Pr-PS
T7Prom	uncleavable support	None	OAS-CPG	Pr-CPG

18 Abbreviations:

19 T7Prom: PCR primer for T7 promoter region;

20 T7Term: PCR primer for T7 terminator region;

21 SIAB: N-succinimidyl-(4-iodoacetyl) aminobenzoate;

22 NHS-PEG-MAL: N-hydroxysuccinimidyl-polyethylene glycol-maleimide, MW2000;

23 MPG: Magnetic Porous Glass;

24 CPG: Controlled-pore Glass;

25 EDA: N-(2-aminoethyl)-3-aminopropylmethyl-dimethoxy-silane;

26 HDA: N-(6-aminohexyl)aminopropyl-trimethoxysilane;

27 OAS, Oligo Affinity Supports;

28 OAS-PS, polystyrene-polyethylene glycol co-polymer;

29 OAS-CPG is a commercially available support (Glen Research , Sterling, Va);

1   PC-E: 5'-phosphorylated DNA primers attached to EDA modified CPG by carbodiimide

2   crosslinking;

3   PC-H: 5'-phosphorylated DNA primers attached to HDA modified CPG by carbodiimide

4   crosslinking;

5   T-PS: T7Term primers synthesized on OAS-PS supports;

6   T-CPG: T7Term primers synthesized on OAS-CPG supports;

7   Pr-PS: T7Prom primers synthesized on OAS-PS supports;

8   Pr-CPG: T7Prom primers synthesized on OAS-CPG supports;

9   E-SIAB: thiol-modified T7Term primer reacted with EDA modified beads via a SIAB crosslinker;

10   H-SIAB: thiol-modified T7Term primer reacted with HDA modified beads via a SIAB crosslinker;

11   E-PEG: thiol-modified T7Term primer reacted with EDA modified beads via a NHS-PEG-MAL

12   crosslinker;

13   H-PEG: thiol-modified T7Term primer reacted with HDA modified beads via a NHS-PEG-MAL

14   crosslinker.

15   **Bead Silanization**

16   As used herein, "dH_2O" refers to water obtained from a Nanopore purification system which

17   was >18 MΩ, UV-sterilized and 0.22 μm filtered prior to use. CPG beads (CPG Inc, Lincoln Park,

18   NJ) having a mean pore diameter of 3.1 μm on particle size of 80/120 mesh, pore volume of 95 cc/g,

19   and surface area of 8.41 m/g were cleaned by immersion in 1:1 concentrated HCl: MeOH for 45

20   minutes, rinsed three times with dH_2O, immersed in concentrated H_2SO_4 for 45 minutes, and rinsed

21   in dH_2O until neutral pH was achieved. The beads were heated in 65°C dH_2O for 45 minutes prior

22   to silanization. Beads were then silanized using N-(2-aminoethyl)-3-aminopropylmethyl-dimethoxy-

23   silane (EDA, Gelest, Tullytown, PA) or N-(6-aminohexyl)-aminopropyltrimethoxysilane (HDA,

24   Gelest). Acid-cleaned beads (typically 50-100 mg) were shaken with 3-5 mls of a 2% solution of

1 EDA or HDA in 25 mM aqueous CH_3COOH for 1 hour at 25°C. [Care must be taken to avoid

2 exposure of the stock silanes to moisture. Thus the silanes were opened, dispensed, and re-sealed

3 in a glovebag under N_2]. The beads were then rinsed 5-10 times with dH_2O (until pH≈5 is attained

4 for the wash solution), then dried at 120°C to ensure the completion of silane film crosslinking

5 (dehydration). The dried beads are then ready for use in **Carbodiimide Crosslinking** or **Treatment**

6 **with Thiolated DNA** as described below.

7 **Carbodiimide Crosslinking**

8   Reactions containing EDA-modified (15.5 mg, 1 ml) or HDA-modified beads (12.5 mg, 0.8

9 ml) were incubated on an orbital shaker with 5 µM 5'-phosphorylated T7Term and 10 mM 1-ethyl-

10 3-(3-dimethylamino-propyl)carbodiimide-HCl (EDC, Sigma Chemical Co, St. Louis, MO) in 10

11 mM 1-methylimidazole (Sigma), pH 7.0, for 3 hours at 50 °C, then 70 hours at 25°C. The beads

12 were then washed briefly twice with dH_2O, then washed twice with 1 ml of 1M NaCl for 1 hour at

13 25°C [to remove non-covalently bound DNA]. 5'-phosphorylated DNA primers attached to EDA

14 or HDA modified CPG by carbodiimide crosslinking are referred to in the text as PC-E and PC-H,

15 respectively (Table 1).

16 **Treatment with Thiolated DNA**

17   EDA- or HDA-silanized beads (3.5-4.0 mg) were treated with one of two heterobifunctional

18 crosslinkers: N-succinimidyl-(4-iodoacteyl) aminobenzoate (SIAB; Pierce, Rockford, IL) or N-

19 hydroxysuccinimidyl-polyethylene glycol-maleimide, MW 2000 (NHS-PEG-MAL; Shearwater

20 Polymers, Huntsville, AL). Crosslinkers were prepared as a 10 mM solution in 1:4 DMSO:MeOH,

21 adding the DMSO first to solubilize the crosslinker. Crosslinkers were opened under N_2 in a

22 glovebag to minimize hydrolysis of the NHS ester. Beads were agitated with crosslinker solution

23 (1.5 ml/3.5-4.0 mg beads) for 2.5 hours at 25°C, then rinsed five times with 1 ml portions of MeOH

24 immediately before coupling to post-PCR thiolated DNA. DNA primers which carried a 5'-disulfide

14

1   modifier were used to generate PCR amplicons (see PCR section, below). PCR reactions (100 μl)

2   were purified using Qiaquick PCR product purification spin columns (Qiagen, Valencia, CA)

3   yielding 50 μl amplicon in Qiagen EB buffer per reaction. For DNA attachment reactions, thiol-

4   modified amplicon was reacted with EDA or HDA modified beads via a SIAB crosslinker (E-SIAB

5   and H-SIAB, respectively) or via a NHS-PEG-MAL crosslinker (E-PEG, H-PEG, respectively).

6   Reactions containing 25μl thiolated amplicon, 1.5 mg beads in HEPES buffer (10 mM HEPES, 1

7   mM EDTA, pH 6.5), and 200 μM tris-(2-carboxyethyl)phosphine (TCEP; Molecular Probes,

8   Eugene, OR) were agitated for 2 hours at 25°C. TCEP rapidly reduces the disulfide modifier on the

9   amplicon to yield free thiol that can react with iodoacetamide or maleimide groups on the

10   crosslinker-modified beads). The beads were then washed with dH_2O several times, followed by

11   incubation in 1M NaCl for 15 hours to remove non-covalently attached DNA, as described in

12   Chrisey, L.A., Lee, G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, 24, 3131-3039.

13   **Oligo Affinity Supports (OAS)**

14   Two different types of oligo affinity supports were obtained from Glen Research: OAS-PS

15   (polystyrene-polyethylene glycol co-polymer) and OAS-CPG. These supports were used with 5'

16   -CE-phosphoramidites (Glen Research) to synthesize oligos in the 5' to 3' -direction, which leaves

17   a free OH group at the 3' end (the 5' -end remains attached to the OAS support). The OAS oligos

18   are not susceptible to cleavage during deprotection but remain tethered to the support. T7Term and

19   T7Prom primers synthesized on OAS-PS and OAS-CPG supports are referred to in the text as T-PS

20   or T-CPG and Pr-PS or Pr-CPG, respectively, where T indicates T7Term and Pr indicates T7Prom

21   primers (**Table I**). The oligo-modified supports were used to prime PCR reactions as described

22   below.

23   **DNA Templates**

24   Plasmids used as templates in this work included pET3a -derived (Novagen, Madison, WI)

1   pET23 (described in Andreadis, J.D. and Black, L. (1998) *J. Biol. Chem., 273, 34075-34086*) and

2   pET6XGFP (described herein). These plasmids all utilize the bacteriophage T7 expression system,

3   which was selected because of its relatively short (17 base pair) consensus promoter sequence and

4   high level of transcription. pET23 contains a 1.56 kB gene encoding the 57 kDa bacteriophage T4

5   Major Capsid Protein (T4 MCP). pETGFP was constructed by isolating the *GFP* gene from p6XHis-

6   GFP (Clontech, Palo Alto, California) via PCR and using primer inserted *Nde*I and *Bam*HI

7   restriction enzyme sites to clone the *GFP* gene into the pET3a expression vector cleaved with the

8   same restriction enzymes. A third plasmid, pTEI and soluble primers complementary to its sequence

9   were used in amplification experiments designed to quantitate non-specific binding to T7-derived

10 immobilized primers.

11 **PCR**

12   The procedure used was a modification of work described in Saiki, R.K., Walsh, P.S.,

13 Lenvenson, C.H. and Erlich, H.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6230-6234, and

14 Rasmussen, S.R., Rasmussen, H.B., Larsen, M.R., Hoff-Jorgensen, R., and Cano, R.J. (1994) *Clin.*

15 *Chem.*, **40**, 200-205. Standard PCR reaction conditions were as follows: $0.1\mu g$ each DNA template

16 (pET6XHISGFP and/or pET23), 1 μM soluble primer A, 50 nM soluble primer B "spike", 30-352

17 μg bead immobilized primer B (as indicated), 20 μM each dNTP, 0.01% BSA, 0.1% Tween 20, 1

18 unit of Taq polymerase (Gibco-BRL, Gaithersburg, MD), 10 mM Tris-HCl pH 8.3 at 25°C, 50 mM

19 KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 0.1% gelatin. In this context, primer A refers

20 to unmodified T7Prom or T7Term oligomers and primer B refers to the bead-immobilized version

21 of these primers as described in **Table 1**. Primer B "spike", refers to the small concentration (50

22 nM) of soluble primer B added to reactions containing mainly an immobilized form of primer B.

23 The use of a soluble spike primer was critical in the initial stages of PCR amplification to increase

24 the ability of the DNA template to interact with bead-immobilized primers that settled in the bottom

1   of the PCR reaction tube. Control reactions in which neither primer was immobilized, and those in

2   which a thiolated primer was used for generation of thiolated amplicons, contained 1 μm of each

3   soluble primer.

4   PCR was initiated under hot start and conditions in a PE thermocycler. PCR was conducted

5   for 35 cycles (each cycle: 1 minute at 94°C, 45 seconds at 66°C, 1 minute at 72°C). The final cycle

6   was followed by an additional 10 minutes at 72°C to ensure complete extension. For some

7   experiments, tubes were removed during the denaturation step and vortexed to resuspend the beads.

8   Subsequent to PCR, the supernatant was removed and beads were washed twice for 30 minutes in

9   150 mM NaCl (30 minutes each wash) and then in nuclease-free dH_2O three times (initially for 1

10   hour, then twice for 15 minutes) with gentle shaking at room temperature. The bead-immobilized

11   DNA templates were then resuspended in 6 μl nuclease-free dH_2O.

12   **Quantitation of Bead-bound DNA**

13   The amount of 1 kb DNA (pETGFP template) specifically attached to beads during

14   simultaneous amplification/immobilization was determined using T4 polynucleotide kinase (PNK,

15   Boehringer Mannheim, Indianapolis, IN) catalyzed incorporation of $[\gamma\text{-}^{32}P]$-dATP (3000Ci/mmole,

16   NEN Life Science Products, Boston, MA) according to manufacturer's 5'-end-labeling protocols.

17   PCR with a soluble and a bead-immobilized primer was performed; the soluble control reaction used

18   a 5'-biotinylated primer and a unmodified primer. After the completion of PCR amplification/

19   immobilization, soluble amplicon from each PCR reaction was purified via Qiaquick PCR

20   purification spin columns (Qiagen) and bead-bound amplicon was washed in 150 mM NaCl twice

21   and rinsed three times with dH_2O. All samples were resuspended in 25 μl dH_2O final volume. These

22   reactions were subsequently treated with T4 PNK (37°C, 1 hour, with gentle shaking) and

23   unincorporated $[\gamma\text{-}^{32}P]$-dATP removed through Qiaquick PCR purification spin columns. Radio-

24   labeled DNA was then eluted with 50 μl dH_2O. In parallel preparation, bead-bound DNA was treated

1　　　with T4 PNK, under the same conditions described above, and washed twice with 150 mM NaCl and

2　　　rinsed three times with dH_2O. Samples were placed in 10 ml Ecoscint H (National Diagnostics,

3　　　Atlanta, GA) and a Packard 1500 Liquid Scintillation Counter (Packard, Downers Grove, IL) was

4　　　utilized to determine $[\gamma\text{-}^{32}P]$-dATP incorporation in the samples. Non-specific binding to CPG or

5　　　PS beads was determined by 5' end-labeling a target gene (pTEI template, target gene approximately

6　　　0.7 kb), whose sequence was non-complementary to the T7-derived immobilized primer. The

7　　　quantity of both soluble and bead-immobilized DNA bound, respectively, was calculated using the

8　　　specific activity of the $[\gamma\text{-}^{32}P]$-dATP. The percentage of total amplicon immobilized during solid-

9　　　phase PCR was determined as the ratio of bead-associated cpm to the total cpm (cpm of bead-bound

10　　DNA plus cpm of soluble DNA from a given PCR reaction). The data reported are the result of

11　　triplicate experiments.

12　　***In vitro* transcription/translation reactions**

13　　　　　　Boehringer Mannheim (Indianapolis, IN) Linked *in vitro* SP6/T7 - Transcription/Translation

14　　Kit (nonradioactive) was used, according to manufacturer protocols, to obtain mRNA transcripts and

15　　protein products using soluble or bead-immobilized DNA. Ambion (Austin, TX) mMessage

16　　mMachine *in vitro* transcription system was used to determine whether bead-immobilized DNA

17　　templates could be recycled or reused. Briefly, DNA immobilized templates were incubated with

18　　transcription reagents for 80 minutes at 37°C with periodic gentle shaking or continuous agitation.

19　　Samples were pelleted briefly (700 x g, 2 minutes, 25°C) and the mRNA transcript-containing

20　　supernatant was removed and analyzed electrophoretically (see **Electrophoretic analysis**). Bead-

21　　immobilized DNA templates were then washed twice with 150 mM NaCl and twice with nuclease-

22　　free dH_2O (30 minutes each with gentle shaking) and fresh transcription reaction reagents added.

23　　This was repeated for a total of seven cycles.

24

1   **Electrophoretic analysis**

2   mRNA transcripts were analyzed on 1-2% agarose gels prepared in 1X MOPS and 6%

3   formaldehyde under RNase-free conditions and electrophoresed at 100V (100-150 mA) for 1 - 1.5

4   hours, as described in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: a*

5   *Laboratory Manual*, 1st ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp

6   7.43 - 7.45. RNA was visualized using ethidium bromide and photographed under UV light.

7   Relative band intensities of the experimental conditions to the control transcript band were

8   determined by scanning the photo into CorelDraw 7 (Corel Corporation) and using NIH Image 1.61

9   (W. Rasband, NIH, Bethesda, MD). For Western blot analysis, protein products were

10   electrophoresed in 12% precast Novex (San Diego, CA) acrylamide gels, electroblotted onto

11   Immobilion-P membranes (Millipore, Billerica, MA), and probed with a 1:1000 dilution of

12   streptavidin-horseradish peroxidase (HRP) conjugate (GIBCO-BRL, Gaithersburg, MD). Stabilized

13   TMB substrate (Promega, WI) was used to visualize the biotinylated protein-streptavidin-HRP

14   complexes.

15

16   **RESULTS AND DISCUSSION**

17   **1. Immobilization of DNA primers**

18   Primers synthesized on OAS supports linked to PS or CPG beads were synthesized in the 5'

19   to 3' direction and remained attached to the PS or CPG support at the 5'-end. Thus, primers

20   synthesized using OAS supports can be used directly as immobilized primers in PCR reactions

21   without further manipulation. A potential disadvantage to the use of OAS supports is an inability

22   to remove failure sequences generated during DNA synthesis. All other primers were purified after

23   synthesis before coupling to beads, to remove failure sequences.

24   In order to be useful for a PCR reaction, bead-bound primers must remain intact during the

1   thermal cycling process. Initially, 5'-thiolated primers attached via heterobifunctional crosslinkers

2   to amine-functionalized CPG were tested, and as expected, and as described in Chrisey, L.A., Lee,

3   G.U., and O'Ferrall, C.E. (1996) *Nucl. Acids Res.*, **24**, 3131-3039, these proved to be heat-labile

4   (data not shown). Thus, a soluble 5-disulfide-modified primer was used to generate thiolated PCR

5   product and immobilization took place as a separate step, subsequent to amplification. The

6   remainder of the immobilized primer chemistries tested were heat stable and thus suitable for solid-

7   phase PCR experiments. **Table 1** summarizes the various combinations of primers, attachment

8   chemistries and beads or polymeric supports that were used in this example.

9   Several amplification conditions were analyzed for the generation of a bead-bound DNA

10   template that was functional for transcription by the bacteriophage T7 polymerase. The primers

11   used in the amplification reactions were as follows (see **Table 1**) : 1) soluble T7 Prom and soluble

12   T7Term, 2) soluble T7Prom and soluble 5'- thiolated T7Term, 3) soluble T7Prom and immobilized

13   T7Term (PC-E, PC-H, T-PS, or T-CPG), and 4) immobilized T7 promoter primer (Pr-PS or Pr-CPG)

14   and soluble T7Term. All PCR and transcription reactions yielded soluble DNA or RNA fragments

15   of the expected size as verified by electrophoretic analysis.

16   The amount of DNA covalently bound to the CPG (PC-E or PC-H), OAS-CPG (T-CPG or

17   Pr-CPG) or PS (T-PS or Pr-PS) beads was determined by 5'-end radiolabeling DNA amplicons

18   obtained following PCR with or without an immobilized primer. A 5' biotin labeled primer was

19   used as one of two primers in the soluble control reaction to limit radiolabeling to just one of the 5'

20   ends of the amplicon and thus, to more closely simulate the situation in which one primer is bead

21   bound. Briefly, the gene for GFP was amplified from pET6XGFP using the following conditions:

22   1) two soluble primers (T7Prom and 5'-biotinylated T7Term), 2) one soluble and one 5'-thiol-

23   modified primer (T7Prom and 5'SH-modified T7Term) or 3) one soluble and one immobilized

24   primer (T7Term and PC-E, PC-H, T-PS, or T-CPG) or (Pr-PS or Pr-CPG and T7Term). The

1   thiolated PCR amplicon generated under condition number 2 above was then immobilized to

2   heterobifunctional crosslinker-modified CPG beads. All bead immobilized templates and soluble

3   PCR fractions were 5'-end labeled as described above. Nonspecific DNA attachment was

4   determined by amplifying a DNA template without any complementarity to the above soluble or

5   immobilized primers, and attempting to radiolabel bead-associated product. Specific covalent

6   attachment to the immobilized primers was determined by the amount of ^{32}P incorporation associated

7   with the beads minus any nonspecific attachment. The specific activity of the [γ^{32}P] -dATP was used

8   to determine the nmol DNA bound per gram beads. **Table 2** compares the yield of DNA PCR

9   amplicons on CPG, OAS-CPG, or OAS-PS beads using each type of immobilized primer, or post-

10 PCR attachment of thiolated amplicons.

11

12 **Table 2.** Quantitation of bead-immobilized DNA

Primer used for immobilization of amplicon	nmol DNA/g bead specific binding
PC-E	141 ± 0.48
PC-H	374 ± 0.39
5'-thiol modified T7Term (⇒E-SIAB)[a]	77 ± 0.75
5'-thiol modified T7Term(⇒H-SIAB)[a]	154 ± 2.5
5'-thiol modified T7Term(⇒E-PEG)[a]	15.4 ± 0.63
5'-thiol modified T7Term(⇒H-PEG)[a]	75.3 ± 0.28
T-PS	25.2 ± 0.6
T-CPG	10.8 ± 0.28
P-PS	18.9 ± 0.84
P-CPG	41.8 ± 1.2

26 Experimental detail is given in the **MATERIALS AND METHODS** section. Bead-bound primers

27 used during the PCR amplification/immobilization protocol are indicated in the far left column using

28 abbreviations described in Table I legend. After PCR amplification, soluble and immobilized DNA

29 were purified separately and labeled with [γ^{32} P]-dATP using T4 polynucleotide kinase at 37°C for

30 one hour. Bound DNA was determined by the specific activities of the oligomers. Non-specific

1   binding was determined by PCR amplification of an approximately 1 kb target gene, whose sequence

2   is non-compatible to the bead-bound primer. [a]The 5'-thiol-modified T7Term primers were used to

3   generate soluble PCR amplicons which were subsequently crosslinked to CPG beads via the

4   chemistry indicated in parentheses. The total soluble thiolated PCR product and the immobilized

5   DNA was radiolabeled as described above to quantitate the amount of PCR product immobilized

6   versus total soluble PCR product. Amplification of a 1 kb target sequence, compatible to the soluble

7   and bead-bound primer, was used to determine specific binding. The data are reported as the mean

8   of triplicate experiments ± standard deviation. Nonspecific binding is calculated as the total binding

9   minus non-specific binding for a particular immobilized primer.

10   The results indicate a wide range of DNA attachment in the nmol DNA/gram bead range for

11   the immobilization chemistries surveyed. It is interesting to note that HDA silanized beads (H-

12   SIAB, H-PEG, and PC-H) consistently yielded higher quantities of covalently attached DNA than

13   EDA silanized beads for all three chemical attachment methods tested. HDA (6 carbons) is a longer

14   silane than EDA (3 carbons) and may be less densely packed on the surface than the EDA. A lower

15   density of silane may provide a greater proportion of silanes that react with the heterobifunctional

16   crosslinkers. DNA template attachment is most successful on beads to which primers were coupled

17   prior to PCR via carbodiimide chemistry (PC-E and PC-H) and those that underwent post PCR

18   immobilization onto HDA crosslinker modified CPG (see PC-E, PC-H, H-SIAB, H-PEG, **Table 2**).

19   The percentage of total PCR product that is bound to the immobilized beads during solid-

20   phase PCR is shown in the following **Table 3**.

21

1   **Table 3.** Percentage of total PCR amplicon immobilized

Solid-phase primer	Percent of total
Non-specific PC	0.45 ± 0.25
PC-E	30.9 ± 0.73
PC-H	60.4 ± 2.2
Non-specific OAS-PS	0.44 ± 0.16
P-PS	3.21 ±. 0.54
T-PS	51.8 ± 2.7
Non-specific OAS-CPG	0.38 ± 0.08
P-CPG	3.04 ± 0.53
T-CPG	16.7 ± 3.5

13   Experimental detail is given in **MATERIALS AND METHODS**, above. Non-specific PC, PS, and

14   CPG refer to non-specific binding to those specific beads. Percentage of total PCR amplicon

15   immobilized was determined by dividing bead bound cpm by total cpm (bead bound cpm and

16   soluble DNA cpm) and multiplying by 100%. Experiments were conducted in triplicate and the

17   data are given as the mean ± the standard error of the mean.

18   As shown in Table 3, the mean bead-bound amplicon generated by PC-E and PC-H

19   immobilized primers (expressed as a percentage of total amplicon) was $30.9 \pm 0.73\%$ and 60.4 ± 2.2

20   %, respectively. Amplification using T-PS and T-CPG solid-phase primers resulted in $51.8 \pm 2.7\%$

21   and $15.6 \pm 3.5\%$ of total amplicon as bound to PS and CPG beads, respectively. Analysis of the four

22   OAS supports indicated a comparatively low mean percentage of total amplicon bound to P-PS and

23   P-CPG immobilized primers ($3.2 \pm 0.54\%$ and $3.0 \pm 0.53\%$, respectively). This result may be

24   sequence-dependent as these primer-bead combinations used the T7Prom primer, which contains

25   a potential hairpin loop sequence that may have interfered with the annealing of the primer to the

26   target molecule.

27   PCR reactions that contained soluble T7Prom and immobilized T7Term primers, but no

28   soluble "spike" of T7Term primer, resulted in undetectable soluble PCR product formation. The

1   addition of a "spike" of soluble T7Term primer was necessary to increase the likelihood of an

2   interaction between soluble coding strand and the bead-bound T7Term primer that settles to the

3   bottom of the PCR tube during amplification. To ascertain whether settlement of the beads during

4   PCR was inhibiting efficient coding strand capture, select reactions were repeated, but the protocol

5   was altered such that the reaction tubes were vortexed vigorously during the denaturation stage of

6   several cycles (2,4,6,8,12, and 24). Contrary to expectations, this resulted in a diminished yield

7   of lower quality transcript (which reached a maximum of 66% of the non-vortexed reaction).

8   All bead-immobilized DNA's tested were functional as templates for *in vitro* transcription.

9   Following the PCR reactions, the 1.56 kB gene encoding the T4 MCP attached to PC-E and PC-H

10   beads were subjected to *in vitro* transcription. Electrophoretic analysis of the mRNA transcripts

11   produced from these reactions shows that although mRNA is produced with each bead-type, PC-E

12   immobilized PCR amplicon is more readily transcribed than DNA attached through PC-H primers.

13   This result was also consistent with *in vitro* transcription results for amplicons produced from 5'

14   thiol-modified primers and subsequently attached to EDA or HDA-modified CPG beads (E-SIAB,

15   H-SIAB, E-PEG, and H-PEG) in that more mRNA was produced when EDA-silanized beads were

16   used. Paradoxically, the observed higher levels of amplicon attachment to HDA silanized beads may

17   be detrimental to efficient *in vitro* transcription of the immobilized template DNA. This may be due

18   to the inability of T7 polymerase and other required transcriptional components to efficiently access

19   and transcribe because of tight DNA packing on and inside the CPG bead pores.

20   A range of concentrations of bead-bound DNA template were examined to establish whether

21   or not a linear relationship exists between the quantity of bead-bound DNA template added to

22   transcription reactions and mRNA produced. A comparison of band intensities of mRNA

23   transcribed from bead-immobilized DNA versus soluble DNA template showed a negligible

24   difference in transcript production above 62 μg beads. The results relative to the Xenopus control

1   (1.00) were 0.65, 0.80, 0.84 for 10, 62, and 124 µg PC-E immobilized DNA, respectively. The

2   results for PC-H relative to the same control are 0.32, 0.28, 0.32 for 10, 62, and 124 ug beads. These

3   observations indicated a near saturated state with little change with increasing bead-bound amplicon

4   concentration. For nearly all of the transcription experiments, reactions were periodically gently

5   agitated during incubation. To determine whether the observed limitation in product formation was

6   due to the restricted accessibility of the immobilized DNA by the transcription reactants as the beads

7   settled to the bottom of the reaction tube, we repeated select experiments and continually agitated

8   during the incubation period. This resulted in an average increase of 19.5% product mRNA (when

9   greater than 50 µg of beads were used per transcription reaction), verifying that exposure of the

10   beads to fresh transcription reagents through mixing improves yield.

11   It is important to note that the relative intensity of the mRNA bands were approximately 80%

12   of the soluble Xenopus DNA transcriptional control, demonstrating the efficiency of the solid-phase

13   templates in *vitro* transcription reactions.

14   A primary interest in developing a solid-phase PCR amplification system using bead-bound

15   primers was for their potential use in repetitive *in vitro* transcription reactions. *In vivo* protein

16   synthesis of polymeric proteins and certain mammalian proteins can be problematic due to the

17   rearrangement or modification of highly repetitive DNA sequences, improper protein folding, and

18   cellular toxicity. Although *in vitro* transcription/translation systems could provide an alternative to

19   *in vivo* protein synthesis, these methodologies can be impractical because of the laborious and often

20   expensive necessity to resynthesize DNA templates after each transcription reaction. Conversely,

21   automated solid-phase peptide synthesis can be quite economical but may be limited by the length

22   and sometimes the sequences of the desired protein. Bead-immobilized DNA templates would

23   provide a convenient way to circumvent these problems by enabling the bead-bound DNA templates

24   to be collected and recycled after each transcription reaction. This approach permits the synthesis

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1   of large proteins not available by automated synthesis and is more economical than conventional *in*

2   *vitro* transcription/translation reactions. Experiments were carried out to determine whether DNA

3   templates, coding for GFP (1.0 kB), immobilized via PC-E and E-PEG crosslinked supports, could

4   be efficient templates for multiple sequential *in vitro* transcription reactions. In these experiments,

5   bead-immobilized PCR amplicons used as templates for *in vitro* transcription, then the immobilized

6   DNA primers were collected by brief centrifugation, rewashed, and exposed to fresh *in vitro*

7   transcription reagents. Significant differences in sequential mRNA production were observed for

8   each type of immobilized DNA template. Although E-PEG yielded high levels of transcript initially,

9   these templates were unable to survive the recycling process beyond 2-3 cycles of repetitive rounds

10   of *in vitro* transcription. This result proved to be the case for most immobilized DNA templates

11   tested with the exception of PC-E immobilized DNA. In this case, the findings indicated that PC-E

12   bound templates can be collected and recycled effectively for use in at least seven repetitive rounds

13   of *in vitro* transcription.

14   **2. Protein production from solid-phase DNA templates**

15   To determine whether mRNA transcripts produced from solid-phase DNA templates were

16   able to initiate translation, reagents for non-radioactive *in vitro* translation were added to the

17   supernatants from *in vitro* transcription reactions. The biotinylated protein products were detected

18   using a streptavidin-HRP conjugate with TMB substrate. A Western blot of biotinylated products

19   produced from solid-phase templates through a coupled *in vitro* transcription/translation system

20   showed that all solid-phase DNA templates produced the desired protein product (57 kDa T4 MCP).

21   A useful feature of the bead immobilization methodology is the ability to amplify and to immobilize

22   either single or multiple DNA target sequences. In a mixing experiment, the genes for GFP and the

23   T4 MCP were amplified via solid-phase PCR using the immobilized PC-E primer and pET6XGFP

24   and pET23 plasmids as templates (either separately or mixed together). The bead immobilized

1   amplicons from each solid-phase PCR reaction (GFP, MCP, GFP + MCP) were collected, washed,

2   and used as templates for *in vitro* transcription/translation reactions. A Western Blot showed the

3   separate production of GFP and the T4 MCP and *in vitro* translation of both proteins simultaneously

4   from a mixture of immobilized templates. Thus, the addition of multiple DNA gene templates to

5   solid-phase PCR reactions yields efficient immobilization of both genes and the subsequent

6   production of multiple protein products.

7   Obviously, many modifications and variations of the present invention are possible in light

8   of the above teachings. It is therefore to be understood that, within the scope of the appended claims,

9   the invention may be practiced otherwise than as specifically described.

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Claims

What is claimed is:

1. A method of making a protein, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second oligonucleotide primers, wherein the first oligonucleotide primer, the second oligonucleotide primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein the first oligonucleotide primer is covalently attached to the solid support,

(b) amplifying the template DNA using the first and second oligonucleotide primers to obtain amplicons covalently attached to the solid support, wherein the amplicons include the transcription and translation regulatory sequences and the protein coding sequence,

(c) transcribing the covalently attached amplicons to obtain an RNA segment that includes the protein coding sequence, and

(d) translating the RNA segment to obtain the protein.

2. A method of making a protein, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second PCR primers, wherein the first PCR primer, the second PCR

primer and/or the template DNA include at least one transcription regulatory

sequence and at least one translation regulatory sequence, and wherein the first PCR

primer is covalently attached to the solid support,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second

PCR primers to obtain amplicons covalently attached to the solid support, wherein the

amplicons include the transcription and translation regulatory sequences and the protein

coding sequence,

(c) transcribing the covalently attached amplicons to obtain an RNA segment that includes

the protein coding sequence, and

(d) translating the RNA segment to obtain the protein.

3. The method of Claim 2 wherein the solid support comprises at least one particle.

4. The method of Claim 2 wherein the solid support comprises at least one particle selected from the

group consisting of glass particles, controlled porous glass particles, magnetic particles, polymeric

particles, hydrogel particles, sol-gel particles and ceramic particles.

5. The method of Claim 3 wherein the solid support comprises at least one particle having a particle

size of about 50 nanometers to 10 mm along an axis of greatest length.

6. The method of Claim 2 wherein the step (a) of providing a solid support having the first PCR

primer covalently attached thereto is accomplished by synthesizing the first PCR primer onto the

solid support.

7. The method of Claim 2 wherein the solid support has a surface that has amine groups, wherein the first PCR primer has phosphate groups, and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished through carbodiimide of coupling the amine groups of the surface of the solid support to the phosphate groups of the first PCR primer.

8. The method of Claim 2 wherein the solid support has a surface that has amine groups, wherein the first PCR primer has amine groups, and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished through carbodiimide coupling of the amine groups of the surface of the solid support to the amine groups of the first PCR primer.

9. The method of Claim 2 wherein the first PCR primer has an amine group and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished by means of a homobifunctional crosslinker that covalently attaches to the solid support and that has an amine-reactive group that reacts with the amine group of the first PCR primer.

10. The method of Claim 2 wherein the first PCR primer has an amine group and wherein step (a) of providing a solid support having the first PCR primer covalently attached thereto is accomplished by means of a heterobifunctional crosslinker that covalently attaches to the solid support and that has an amine-reactive group that reacts with the amine group of the first PCR primer.

11. The method of Claim 2 wherein the first PCR primer is between 5 and 500 bases long.

12. The method of Claim 2 wherein the first PCR primer includes regulatory sequences from bacterial, viral, fungal, mammalian or insect cell expression systems.

13. The method of Claim 2 wherein the first PCR primer includes a T7 transcription promoter sequence.

14. The method of Claim 2 wherein at the conclusion of step (d), the solid support is separated from the protein produced in step(d), and steps (c) and (d) are repeated at least one additional time.

15. A method of making a protein, the method comprising the steps of:

    (a) providing

        (i) a solid support,

        (ii) a template DNA that includes at least one sequence that encodes a protein, and

        (iii) first and second PCR primers, wherein the first PCR primer, the second PCR primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence,

    (b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons that include the transcription and translation regulatory sequences and the protein coding sequence,

    (c) covalently attaching the amplicons to the solid support,

    (d) transcribing the covalently attached amplicons to obtain an RNA segment that includes the protein coding sequence, and

    (e) translating the RNA segment to obtain the protein.

16. A method of making a plurality of different proteins simultaneously, the method comprising the steps of:

    (a) providing

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(i) a solid support,

(ii) a template DNA that includes a plurality of different coding sequence that each encodes a different protein, and

(iii) first and second PCR primers, wherein the first PCR primer, the second PCR primer and/or the template DNA include at least one transcription regulatory sequence and at least one translation regulatory sequence, and wherein the first PCR primer is covalently attached to the solid support,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second PCR primers to obtain amplicons covalently attached to the solid support, wherein the amplicons includes a transcription regulatory sequence and the plurality of different protein coding sequence,

(c) transcribing the covalently attached amplicons to obtain a plurality of different RNA segments that each include a protein coding sequence, and

(d) translating the plurality of different RNA segments to obtain a plurality of different proteins.

17. A method of making a plurality of different proteins simultaneously, the method comprising the steps of:

(a) providing

(i) a solid support,

(ii) a plurality of different template DNAs, each including a sequence that encodes a different protein, and

(iii) a first PCR primer that is covalently attached to the solid support and that hybridizes with each of the different template DNAs, and

(iv) a plurality of different second PCR primers, each hybridizing with a different

template DNA,

wherein either the first PCR primer or the second PCR primers or the template DNA

includes at least one transcription regulatory sequence and at least one translation

regulatory sequence,

(b) amplifying the plurality of different template DNA by the polymerase chain reaction using

the first and second PCR primers to obtain a plurality of different amplicons covalently

attached to the solid support, wherein each amplicons includes a transcription regulatory

sequence and a protein coding sequence,

(c) transcribing the plurality of different covalently attached amplicons to obtain a plurality

of different RNA segments that each include a protein coding sequence, and

(d) translating the plurality of different RNA segments to obtain a plurality of different

proteins.

18. A method of preparing and delivering DNA to an organism, wherein the DNA has at least one

protein encoding sequence, at least one transcription regulatory sequence and at least one translation

regulatory sequence, the method comprising the steps of:

(a) providing

(i) a solid support, wherein the solid support comprises particles having a size

sufficiently small to be injected into an organism,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second oligonucleotide primers, wherein the first oligonucleotide

primer, the second oligonucleotide primer and/or the template DNA include at least

one transcription regulatory sequence and at least one translation regulatory

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sequence, and wherein molecules of the first oligonucleotide primer are covalently

attached to the particles,

(b) amplifying the template DNA using the first and second oligonucleotide primers to

obtain amplicons covalently attached to the particles, wherein the amplicons include the

transcription and translation regulatory sequences and the protein coding sequence,

(c) combining the particles having covalently attached amplicons with a pharmaceutically

acceptable carrier, and

(d) injecting the particles into an organism.

19. A method of preparing and delivering DNA to an organism, wherein the DNA has at least one

protein encoding sequence, at least one transcription regulatory sequence and at least one translation

regulatory sequence, the method comprising the steps of:

(a) providing

(i) a solid support, wherein the solid support comprises particles having a size

sufficiently small to be injected into an organism,

(ii) a template DNA that includes at least one sequence that encodes a protein, and

(iii) first and second PCR primers, wherein the first PCR primer, the second PCR

primer and/or the template DNA include at least one transcription regulatory

sequence and at least one translation regulatory sequence, and wherein molecules of

the first PCR primer are covalently attached to the particles,

(b) amplifying the template DNA by the polymerase chain reaction using the first and second

PCR primers to obtain amplicons covalently attached to the particles, wherein the amplicons

include the transcription and translation regulatory sequences and the protein coding

sequence,

(c) combining the particles having covalently attached amplicons with a pharmaceutically acceptable carrier, and

(d) injecting the particles into an organism.

20. A device for making a protein, the device comprising

(i) a solid support, and

(ii) a DNA segment having a first end and a second end, wherein the first end is covalently attached to the solid support, wherein the second end contains phosphorothioate linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence.

21. A pharmaceutical composition comprising

(i) a plurality of particles, the particles having a size sufficiently small to be injected into an organism,

(ii) a plurality of molecules of a DNA segment, wherein the DNA segment has a first end and a second end, wherein the first end is covalently attached to the particles, wherein the second end contains phosphorothioate linkages, and wherein the DNA segment includes at least one protein coding sequence, at least one transcription regulatory sequence and at least one translation regulatory sequence, and

(iii) a pharmaceutically acceptable carrier.

International application No.
PCT/US00/34426

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; A61K 48/00; C07H 21/04; C12P 19/34
US CL :435/6, 69.1, 91.1, 91.3; 514/2, 44; 536/23.1, 25.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 91.1, 91.3; 514/2, 44; 536/23.1, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, EMBASE, CAPLUS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,571,690 A (HECHT et al.) 05 November 1996, see entire document.	1-21
Y	US 5,665,563 A (BECKLER) 09 September 1997, see entire document.	1-21
Y	US 5,700,667 A (MARBLE et al.) 23 December 1997, see entire document.	1-21
Y	BEATTIE et al. Hybridization of DNA Targets to Glass-Tethered Oligonucleotide Probes. Molecular Biotechnology. 1995, vol. 4, pages 213-225, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 MARCH 2001	01 JUN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ANDREW WANG
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Form PCT/ISA/210 (second sheet) (July 1998) *

International application No.
PCT/US00/34426

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	ANDREADIS et al. Use of immobilized PCR primers to generate covalently immobilized DNAs for in vitro transcription, translation reactions. Nucleic Acids Research. 2000, vol. 28, no 2, pages i-viii, see entire document.	1-21
Y	MARBLE et al. RNA Transcription from Immobilized DNA Templates. Biotechnol. Prog. 1995, vol. 11, pages 393-396, see entire document.	1-21